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Antimicrobial activities of aldehydes and ketones produced during rapid volatilization of biogenic oils

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ANTIMICROBIAL ACTIVITIES OF ALDEHYDES AND KETONES PRODUCED DURING RAPID VOLATILIZATION OF BIOGENIC OILS

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ABSTRACT

A petroleum middle distillate known as the Fog oil (FO) has been used in the US military battle field to create obscurant smoke screens. During studies on the feasibility of replacing FO with relatively environmentally benign natural oil esters, with similar flow properties, such as the methyl soyate (MS) it was observed that FO and MS aerosols and vapors, produced through a thermal process, were lethal to *Salmonella typhimirium* strains (Ames strains) used for mutagenic activity(Modified Ames Assay) even after very short exposures. Further studies carried out at the Center of Environmental Science and Technology- University of Missouri Rolla showed that vapors produced from the vegetable oil esters under certain conditions exhibited high antimicrobial activity against wide range of Gram-positive and Gram-negative bacteria. A gas chromatographic-mass spectrometric analysis of the vapors showed that the presence contained a number of hydrocarbons, short chain acids, aldehydes and ketones.

A part of this thesis deals with an assessment of antimicrobial properties of the aldehydes and ketones detected in vegetable oil ester vapors. Experiments involved introduction of a known amount of individual aldehyde and ketone into an exposure chamber containing Petri dishes with nutrient agar inoculated with *Salmonella typhimurium*. Petri dishes were removed from the chamber after varied exposure periods. Gas phase concentrations of the test chemical were monitored with gas chromatography. The results of the experiments showed that individual aldehydes and ketones exhibited much lower antimicrobial activity than the vapors produced during thermal aerosol generation process.

Another set of experiments showed that the vapors generated from methyl ester were very effective against bacterial Biofilms produced by *Staphylococcus epidermidis* and *Klebsiella pneumonia.*

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1. INTRODUCTION AND REVIEW OF LITERATURE

1.1 DISINFECTANTS

In recent years, there has been a growing awareness of the need for the development of new and safe antimicrobial agents against contamination by various microorganisms in different areas like medical devices, food industry, feed supplies and storage spaces eg., by *Escherichia coli*, *Salmonella enteriditis and Staphylococcus epidermidis* which has led in increased use of disinfectants and antiseptics by the general public [1].

Disinfectants are antimicrobial agents that are applied to non-living objects to destroy microorganisms, in a process which is known as disinfection. Disinfectants should generally be distinguished from antibiotics that destroy or inhibit microorganisms within the body, and from antiseptics, which destroy microorganisms on living tissue. Sanitizers are high level disinfectants that kill over 99.9% of a target microorganism in applicable situations. Very few disinfectants and sanitizers can sterilize (complete elimination of all microorganisms). Those treatments that can sterilize depend entirely on their mode of action. Disinfectants are abundantly used in hospitals laboratories and other health care facilities to treat different surfaces. There are different types of disinfectants from various sources; perfume oils (mixture of natural essential oils and odoriferous organic chemicals) were active in destroying microorganisms either by direct contact with the oils or their vapors [2], volatile compounds like trans-2-hexenal, 2,4-hexadienal, furfural, β-ionone, and 1-nonanal found to occur naturally in corn ears inhibit the growth of *Aspergillus flavus* [3], *in vitro* studies of terpeneless oils like cinnamon, caraway, dill

and anise found to possess great antifungal activity on 10 organisms both pathogenic and nonpathogenic fungal cultures [4] and isolation of nonisoprenoid alkyl side chain phenolic compounds such as anacardic acids, cardols, methylcardols and cardanols from cashew *Anacardium occidentale* (Anacardiaceae) apple found to have antimicrobial activity on *Bacllus subtilis, Staphylococcus, Streptococcus mutans and Pseudomonas aeruginosa* [5]. The chemical disinfectants (or' biocides') are broadly classified as phenolic compounds, alcohols, quaternary ammonium compounds, chlorine compounds, aldehydes, halogenated tertiary amines, hydrogen peroxide and gases like ethylene oxide with different mode of action which remain largely unknown. The above listed biocides are effective against wide range of bacteria both gram positive and negative strains.

Characteristics of ideal disinfectant are listed below.

- Broad spectrum antimicrobial activity
- Solubility
- Stability
- Innocuous to humans and other animals
- Homogeneity
- Non-combination with extraneous organic material
- Toxicity to microorganisms at room or body temperature
- Capacity to penetrate
- Deodorizing ability
- Inexpensive
- Non- corrosive
- Availability

Some disinfectants have a broad spectrum (kill nearly all microorganisms) while others kill a smaller range of disease-causing organisms but are preferred for other properties (they may be non-corrosive, non-toxic, or inexpensive).Thus no one disinfectant meets all these characteristics. Many factors account for their activity which include the condition of the surface, concentration of the chemical compounds, temperature, pH, and mineral interference with active ingredients, type of microbial contamination and the physical composition of the surface. Disinfectant activity of different compounds seems to depend on the polarity of the functional groups [9]. Alcohols, fatty acids and aldehydes possess a polar functionality in the molecule, which imparts the anti-microbial activity of the molecule. Kabara *et al*. [10] and Ouattara [11] correlated the antibacterial activity to the balance between the hydrophobic and hydrophilic groups in the molecules e.g. the activity of the compounds strongly depends on its chain length. The antimicrobial activity of long-chain aldehydes and alcohols (>C6) have also been found to be related to the balance between the polar (hydrophilic) and nonpolar(hydrophobic) portions of the molecules[12]. The toxicity of the volatile aldehydes is dependent on their antimicrobial.

1.2. SOME COMMONLY USED CHEMICAL DISINFECTANTS

There are several classes of chemicals used as disinfectants. Many of these disinfectants kill a wide range of bacteria and in general are called antibacterials. Antibacterials may be divided into two groups according to their speed of action and

residue production: The first group contains those that act rapidly to destroy bacteria, but quickly disappear (by evaporation or breakdown) and leave no active residue behind (referred to as *non-residue-producing*). Examples of this type are the alcohols, chlorine, peroxides, and aldehydes. The second group consists mostly of newer compounds that leave long-acting residues on the surface to be disinfected and thus have a prolonged action (referred to as *residue-producing*). Common examples of this group are triclosan, triclocarban, and benzalkonium chloride. All products that claim to kill bacteria and/or viruses have some kind of antibacterial agent. Alcohols, chlorine and peroxides have been used for many decades in health-care and cleaning products. Within the past two decades, the residue-producing antibacterials [Table 1.1] once used almost exclusively in health care institutions have been added to increasing numbers of household products, particularly soaps and cleaning agents. A recent survey reported that 76% of liquid soaps from 10 states in the US contained triclosan and approximately 30% of bar soaps contained triclocarban. Many cleaning compounds contain quaternary ammonium compounds. Because these compounds have very long chemical names, they are often not easily recognized as antibacterial agents on packaging labels. More recently, triclosan has been bonded into the surface of many different products with which humans come into contact, such as plastic kitchen tools, cutting boards, highchairs, toys, bedding and other fabrics[6].

ALCOHOLS: Alcohols exhibit rapid broad spectrum anti-microbial activity against bacteria, viruses and fungi. However, due to the fact that they lack sporidical activity alcohols are used more as disinfectant on hard surfaces and as antiseptics on

skin for sterilization. Isopropyl alcohol is considered slightly more efficacious against bacteria and ethyl alcohol is more potent against viruses because of its greater hydrophilic properties [7].

ALDEHYDES: Amongst the aldehydes, glutaraldehyde and formaldehyde are the most commonly used disinfectants and sterilants. Glutaraldehyde is an important dialdehyde used as sterilant and disinfectant, in particular for low-temperature disinfection of endoscopes and various surgical instruments. Glutaraldehyde has a broad spectrum of activity against bacteria and their spores, fungi, and viruses. Formaldehyde is a mono-aldehyde that exists as a freely water-soluble gas also named as formalin. Formalin is an aqueous solution containing 34 to 38% (wt/wt) CH_2O with methanol to delay polymerization. It is bactericidal, sporicidal and virucidal but works more slowly than glutaraldehyde [8].

ETHYLENE OXIDE: Ethylene oxide is another effective disinfectant; however it is mutagenic and there is a risk of explosion [7] but it is good for use with sensitive instruments since the toxic residues are easy to remove with aeration.

PHENOLS: Phenol- type antimicrobial agents have long been used for their antiseptic, disinfectant, or preservative properties, which process they are used for depends on the compounds. For example, cresols are an important group of phenolic and are the active ingredient in Lysol brand disinfectant. Chlorhexidine is a related compound that, when combined with detergent or alcohol, is used to clean surgical devices [13].

Bis-Phenols are hydroxyl-halogenated derivatives of two phenolic groups connected by various bridges [14]. They exhibit broad-spectrum efficacy but have little activity against *Pseudomonas aeruginosa* and molds and are sporostatic toward bacterial spores. Triclosan and hexachlorophene are the most widely used biocides in this group, especially in antiseptic soaps and hand rinses. Both compounds have been shown to have cumulative and persistent effects on the skin [7]. Triclosan exhibits activity against gram-negative, gram-positive and yeast with more activity against gram-positive than gram-positive. Reports have also suggested that in addition to its antibacterial properties, Triclosan may have anti-inflammatory activity [15]. Despite the, broad-spectrum efficacy of hexachlorophene, concerns about toxicity, in particular in neonates have meant that its use in antiseptic products has been limited [7].

QUATERNARY AMMONIUM COMPOUNDS: Quaternary ammonium compounds are cationic surfactants which are the most useful antiseptics and disinfectants for a variety of clinical purposes(preoperative disinfection of unbroken skin, application to mucous membranes, and disinfection of noncritical surfaces) [16]. They are sometimes known as cationic detergents.

HALOGEN –RELEASING AGENTS: Chlorine and iodine based compounds are the most significant microbicidal halogens that have been traditionally used for antiseptic and disinfectant purposes. Chlorine releasing agents (CRA) are widely used for hard surface disinfection and disinfecting spillages of blood containing human immunodeficiency virus (HIV) [17]. CRAs at higher concentrations are sporicidal [18]. The sporicidal activity depends on the pH and concentration of available chlorine.

Olivieri et.al showed that CRAs also possess virucidal activity [19]. Alcoholic solutions of iodine have been used for 150 years as antiseptics with bactericidal, fungicidal, tuberculocidal, virucidal and sporicidal properties (20).In order to overcome the disadvantage of iodine solutions which are unstable, stable iodophores (iodine carriers) were developed.

PEROXYGENS: Hydrogen peroxide H_2O_2 is a widely used biocide for disinfection, sterilization and antisepsis. Although pure solutions are generally stable, most contain stabilizers to prevent decompositions. H_2O_2 demonstrates broad spectrum efficacy against viruses, bacteria, yeasts and bacterial spores [21].Greater activity is seen against gram-positive than gram-negative bacteria; however, the presence of catalase or other peroxidases in these organisms can increase tolerance in the presence of lower concentrations of perioxide. Higher concentrations of H_2O_2 (10%-30%) and longer contact times are required for sporicidal activity [22]. Peracetic acid "(PAA) is considered a more potent biocide than hydrogen peroxide when used at lower concentrations and is also sporicidal, bactericidal, virucidal and fungicidal. Its main application is as a low temperature liquid sterilant for medical devices, flexible scopes and hemodialyzers [23].

Table 1.1 List of some disinfectants.

Non-residue- producing antibacterials	
Substance group	*Substance*
Alcohols	Ethanol, Isopropanol
Aldehydes	Glutaraldehyde, formaldehyde
Halogen-releasing compounds	Chlorine compounds, Iodine compounds
Peroxides	Hydrogen peroxide, ozone, peracetic acid
Gaseous substances	Ethylene oxide, formaldehyde
Residue- producing antibacterials	
Anilides	Triclocarban
Biguanides	Chlorhexidine, alexidine, polymeric biguanides
Bisphenols	Triclosan, hexachlorophene
Halophenols	PCMX (p-chloro-m-xylenol)
Heavy metals	Silver compounds, mercury compounds
Phenols and Cresols	Phenol, cresol
Quaternary ammonium compounds	Cetrimide, benzalkonium chloride, Cetylpyridinium chloride

1.3. MECHANISM OF DISINFECTION

Disinfection processes have been classified into different level according to the CDC's "Guideline for Hand washing and Hospital Environmental Control" as sterilization, high-level disinfection, intermediate-level disinfection, and low-level disinfection [6,7]. In these classification are sterilization is the destruction of all microorganisms, including bacterial spores. High–level disinfection is expected to

destroy all microorganisms with the exception of bacterial spores. Intermediate-level disinfection inactivates *Mycobacterium tuberculosis*, vegetative bacteria, most viruses and most fungi, but it does not necessarily kill bacterial spores. Low-level disinfection inactivates vegetative bacteria, most viruses and most fungi, but it cannot be relied upon to kill resistant microorganisms such as M. *tuberculosis* or bacterial spores.

Considerable effort has been invested in unraveling the mysteries behind the mechanism of disinfectant action. There are some common sequences of events that take place in any type of cell or entity when exposed to disinfectant compounds. This can be envisaged as interaction of the disinfectant with the cell surface followed by penetration into the cell and action at the target site. This sequence might differ from cell to cell due to the nature and composition of the surface of the cell, which is also vulnerable to changes in the environment. The specific mode of action of alcohols which are widely used is little known although it is generally believed that they cause membrane damage and rapid denaturation of proteins, protein leakage from the cell [24]. Subsequently interfere with cell metabolism and results in cell lysis [25].

Aldehydes (mainly glutaraldehyde) possessing high microbicidal activity have been shown to associate with the outer layer of bacterial cells [26], specifically with unprotonated amines on the cell surface. This in turn shows an inhibitory action on transport of ions across the cell wall and on enzyme systems where access of substrate to enzyme is prohibited [27]. Glutaraldehyde is more active at alkaline than acidic pH. As external pH is altered from acidic to alkaline more reactive sites will be formed at the cell surface, leading to a more rapid bactericidal effect .The dialdehyde and glutaraldehyde is

sporicidal at both alkaline and acidic pH strongly interacts with outer spore layers [28]. Acidic glutaraldehyde interacts with and remains at the cell surface whereas alkaline glutaraldehyde penetrates more deeply into the spore coat [29]. Phenol induces progressive leakage of intracellular constituents, including K^+, the first index of membrane disintegration [30] Srivatsava and Thompson [31] proposed that phenol acts only at the point of separation of pairs of daughters cells with young bacterial cells being more sensitive than older cells. Another class of disinfectants, Bisphenols, have been suggested to inhibit the uptake of essential nutrients and higher concentrations release the cellular contents which result in cell death. Quaternary ammonium compounds are membrane active agents. Salton *et.al* [32] proposed the schematic path of action involving the adsorption and penetration of QAC. This is followed by reaction of QAC with cytoplasmic membrane followed by disorganization of membrane, degradation of proteins and nucleic acids ending with wall lysis caused by autolytic enzymes. Thus results in loss of structural organization and integrity of the cytoplasmic membrane [33]. Halogen Releasing Agents are highly active oxidizing agents and thereby destroy the cellular activity of proteins. Hypochlorous acid has also been found to disrupt oxidative phosphorlyation [34] and its membrane associated activity. The mode of action is also similar to chlorine releasing agents by attacking surface proteins and also destabilizing membrane fatty acids. H_2O_2 acts as an oxidant by producing hydroxyl free radicals which attack essentials cell components, including lipid, proteins and DNA. It has been proposed that exposed sulfhydryl groups and double bonds are particularly targeted [35].

1.4 FATTY ACID ESTERS AND BIOGENIC OILS

Examination of literature shows that fatty acids, esters and oils exhibit antibacterial and antifungal properties. They may not be as effective as aldehydes and ethylene oxide but may not be as toxic or harmful as commercially available disinfectants. A study of the antimicrobial effect of fatty acids showed the antimicrobial effect is significant in the case of straight chain fatty acids [36]. Esters of fatty acids with 6-18 carbons such as with glycerol caprylate (C_{10}), glycerol laurate (C_{12}) and glycerol mysristate (C_{14}) exhibit significant antibacterial, anti yeast and anti mold activity [37,38]. The relative antimicrobial activity of fatty acids and glycerol esters against *Bacillus cereus* was found to decease in the order: stearic <oleic< lauric<glycerol laurate < linolenic acid [39]. Free lauric acid (C_{12} acid) was found to be most effective against gram positive organisms [40]. Plots of ln (Minimum Inhibitory Concentration) versus fatty acid chain length are generally parabolic. The antimicrobial activity increases with chain length up to a point and then decrease with further increase in chain length. Long chain fatty acid also acts as inhibitors of DNA methyl transferases which are known to be important for methylation in bacteria [41] .The antibacterial ability of fatty acid is probably due to the ability of these compounds to disrupt the membranes of bacterial cells leading to cell lysis [42].

Short chain aldehydes are natural flavor and fragrance constituents that act as anti-microbial agents, delaying or preventing onset of decay [43, 44]. 2-hexenal is noted to possess broad-spectrum antimicrobial activity. Although the precise mode of action of alkenals is not yet known, these likely permeate through passive diffusion across the

plasma membrane. Once inside the cells, the aldehyde moiety readily reacts with biologically important nucleophilic groups like nucleic acid bases of a DNA strand, thiols, amides and carboxyls [45]. It has been shown that antibacterial activity against *Salmonella choleraesuis* increase with the addition of CH_2 group up to (2E)-dodecenal. The change in activity is most likely related to a balance between the hydrophilicity of the unsaturated aldehyde subunit and the hydrophobicity of the alkyl portion of the molecule similar to their action against *Saccharomyces cerevisiae* [46]. Gueldner *et al*. (1985) demonstrated that the volatiles, (2E)-hexenal, 2, 4-hexadienal, 1-hexanol, furfural, β-ionone, 1-nonanol and some synthetic compounds inhibited the growth of *Aspergillus flavus*. The antibacterial activity of these compounds was highest for the aldehydes followed by the ketones and then the alcohols[3]. Cocciaoni *et al* reported that compounds such as esters, aldehydes, terpenes, alcohols and hydrocarbons are more effective antimicrobial agents when used in the vapor phase than in the solution phase [47]. Moreover many of these when employed simultaneously as a mixture may yield synergistic effect resulting in better disinfection.

Antibacterial activity of oils and their constituents has been known for many years. An *in vitro* study showed that among volatile oils the highest antibacterial activity was observed with eucalyptus, birch tar, cinnamon, balsam of tolu and cedar leaf oils. *Bacillus subtilis* was shown to be the most susceptible organism to the volatile oils while *Aerobacter aerogenes* was known to be the most resistant [48]. Greatest sum totals of zone of inhibition was observed while testing antifungal properties of essential oils like

oregano, lemon grass, thyme, sweet birch, coriander and cinnamon. However the mode of action of these volatile oils is not yet known.

1.5 METHYL SOYATE DERIVED VAPORS AS DISINFECTANT AND STUDIES WITH INDIVIDUAL ANALYTE FROM THE VAPOR

Aerosols generated through volatilization and subsequent recondensation of oil vapors have been used as obscurant (smoke) screens during military operations since the early twentieth century. The battlefield obscurant used for impairing view in the visible region produce a dense white cloud which attenuates light transmission in the visible region of the electromagnetic spectrum thereby confounding enemy sensors and smart munitions. For the past four decades, fog oil [de-aromatized middle distillate petroleum (MIL-F-12070E)] has been used by US military as obscurant in the battle field and during training exercises. Recently use of biogenic (vegetable) oils has been investigated as a substitute for the fog oil. Biogenic oils are non-petroleum based oils with physical properties similar to those of the fog oil in addition these oils are free of potentially carcinogenic poly aromatic hydrocarbons (PAHs). During these evaluations the fog oil and vegetable oil esters and their vapors were subjected to the Ames test [62]. The results of the Ames test revealed that the vapors obtained from oils under certain conditions were not mutagenic but lethal to bacterial strains used in the Ames test. These results opened up the potential use of oil derived vapors as antimicrobial agents. Disinfectant activity studies with vapor had showed very effective antimicrobial activity at very small time intervals. Simultaneous studies in analysis of the smoke unraveled the chemical composition of the aerosols in the smoke. Individual chemicals identified were studied for their antimicrobial activity in comparison with relative activity of the whole smoke.

1.6. BIOFILMS

A biofilm is complex aggregation of microorganisms marked by the excretion of a protective and adhesive matrix. Biofilms are also often characterized by surface attachment, complex community interactions and extracellular matrix of polymeric substances. Formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. These first colonists adhere to the surface initially through weak, reversible van der Waals forces. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion molecules such as pili. The first colonists facilitate the arrival of other cells by providing more diverse adhesion sites and beginning to build the matrix that holds the biofilm together. Some species are not able to attach to a surface on their own but are often able to anchor themselves to the matrix or directly to earlier colonists. It is during this colonization that the cells are able to communicate via quorum sensing. Once colonization has begun, the biofilm grows through a combination of cell division and recruitment. The final stage of biofilm formation is known as development, and is the stage in which the biofilm is established and may only change in shape and size. This development of biofilm allows for the cells to become more antibiotic resistant [64].

Biofilm problems are present in different industrial and medical fields, such as dentistry, drinking water, cooling water, oil recovery, food processing, paper manufacturing, ship hulls, and medical implants [49]. Biofilms are the most common mode of bacterial growth in nature and are highly resistant to antibiotics (relevant in clinical infections). This form of microbial growth has been studied in a wide range of

scientific disciplines including biomedicine, water engineering and evolutionary biology [50].A large number of studies have been performed targeted at the bacterial biofilms [51]. Transplantation procedures, immunosuppression, the use of chronic indwelling devices and prolonged intensive care unit stays have increased the prevalence of bacterial as well as fungal diseases due to biofilms[52]

Bloodstream infections (BSI) are a significant cause of morbidity and mortality in hospitalized patients [53].The use of central venous catheters (CVCs) in current therapeutic practice has been found to be responsible for more than 90% of these BSI [54], resulting in 20-30% mortality among hospitalized patients with CVC-related BSI (CR-BSI) [55].The microorganisms most commonly associated with CR-BSI are *Staphylococcus aureus*, *Candida albicans*, coagulase-negative S*taphylococci*, and aerobic gram-negative bacilli [52].

The pathogenesis of most CR-BSI is complex and multifactorial, and in various studies has been linked to (a) duration of catheter retention (short-term, <14 days; or long-term, >14 days), (b) type of catheter material (Teflon, silicone elastomer, polyurethane, etc.), and (c) adherence/colonization properties of the organism [56]. In one study, direct microscopic examination by scanning electron microscopy showed that extensive biofilms were formed on 88% of CVCs that had been placed in intensive care unit patients [57]. Biofilms are phenotypically different from planktonic (suspended) cells [50], and the most important manifestation of these differences is a significantly decreased susceptibility of biofilms to antimicrobials, a feature found to be true for both bacterial and fungal [58] biofilms.

1.7 RESISTIVITY OF BIOFILMS

Bacterial biofilms formed by *Pseudomonas aeruginosa, Pseudomonas fluorescens*, *Escherichia coli*, and *Vibrio cholerae* have been studied in some detail [50]. A number of mechanisms including (a) drug efflux pumps [59], (b) drug diffusion [60], and (c) penetration through the extra cellular matrix (ECM) [61] have been proposed as mechanisms for the resistance of bacteria growing as biofilms [62]. However, none of these mechanisms alone can explain the phenomenon of increased resistance associated with biofilms. Other studies have identified genetic components required to form single-species bacterial biofilms and resulted in the identification of quorum sensing signals in *P. aeruginosa* biofilms [50].

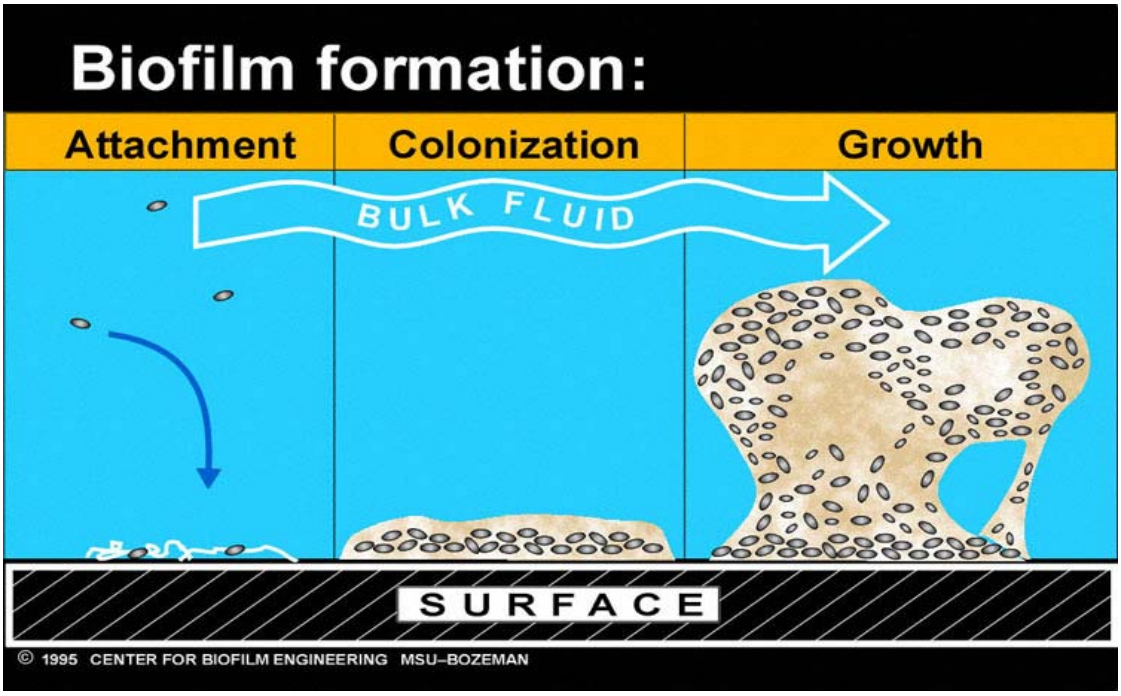


Figure 1.1. 3 stages of biofilm formation: attachment, colonization and growth of complex biofilm structures. *Sited from Center of Biofilms Engineering MSU-Bozeman*

The current study was undertaken to expand on research initiated at the Center for Environmental Science and Technology- University of Missouri- Rolla towards the development of a highly effective wide spectrum vapor disinfectant from innocuous, renewable and biodegradable natural substances. The oxidized-oil chemical vapor disinfectant has shown to be of a great potential at the bench-scale. It is found to be effective against *Mycobacterium smegmatis, Mycobacterium phlei, Salmonella typhimurium, Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Enterobacter cloacae, Shigella sonnei, Serratia marcescens, Bacillus subtilis, Bacillus stearothermophilus, Staphylococcus aureus, Staphylococcus eperdermis, and Sterptococcus mutans* [63]. Disinfectant activity of vapor generated from biogenic oil on biofilms produced from bacterial strains *Klebsiella pneumoniae* and *Staphylococcus epidermidis* was tested. The results may open a way for new safe and effective disinfectants for bacterial biofilms.

2. STATEMENT OF OBJECTIVES

The overall objective of the research presented in this thesis was to evaluate the antimicrobial properties of commercially available neat compounds in order to validate the analytes which were found in the smoke generated during rapid volatilization of biogenic oil esters and mineral oils. To attain this objective various analytes were selected from the analytical data provided for the smoke, and neat compounds were purchased. A special desiccator was designed to conduct experiments. Simultaneously vapor phase concentration of the neat compounds was determined to establish relation between neat compounds and the analytes found in smoke. Other part of the thesis includes evaluation of the antimicrobial property of volatiles produced during thermal oxidation of biogenic oil esters and mineral oil on Biofilms.

3. EXPERIMENTAL

3.1 DISINFECTION ACTIVITY OF VOLATILE COMPOUNDS

A small bench scale setup was built in order to measure the disinfection properties of the aldehydes and ketones discovered in the vapors generated by rapid volatilization of methylsoyate (MS). In order to test their antimicrobial activity of the neat compounds a glass vacuum desiccator without desiccant was used as the container in which the bacterial strain were exposed to the compounds. The desiccator was sealed with vacuum grease applied well to the flanges. All the experiments were conducted at room temperature under an air flow hood as a safety measure. Hamilton syringes capable of holding different volumes ranging from 10µL, 25µL, and 100µL were used to inject the samples into the desiccator via an outlet in the lid. Glass and aluminum desiccators with different volumes of 3, 4.5 and 6.5 liters were used as exposure chambers. Neat compounds were used without any solvent for exposure studies.

3.1.1 Materials

3.1.1.1 Neat Compounds. Compounds listed in the table 3.1 with specific concentrations were found in the aforementioned study were used to test antimicrobial activity against bacteria. High purity Standards (98%) of acetaldehyde, propanal, butanal, pentanal, hexanal, and hexanone were purchased from Sigma-Aldrich, St Louis, MO. Heptanone was purchased from M.P Biochemical's, Inc Ohio. All above listed chemicals were properly stored in order to avoid any contamination and vaporization before experiments.

Table 3.1List of chemicals found in the Methyl soyate vapor

Compounds	Concentration(µg/L) found in earlier study
Acetaldehyde	25.71µg/L
Propanal	108.40µg/L
Butanal	60.29 µg/L
Pentanal	65.21µg/L
Hexanal	160.80µg/L
Heptanal	79.42µg/L
Hexanone	12.6µg/L
Heptanone	12.2µg/L

3.1.1.2. Nutrient medium for bioassay. Tryptic soy agar (1.5% agar, 3% tryptic soy broth) and tryptic soy broth (3% tryptic soy broth) were used for the anti-microbial assay. The agar was purchased from Fisher Scientific (Fair lawn, NJ). The tryptic soy broth (soybean-casein digest medium) was purchased from Becton, Dickinson and company (Sparks, MD).

3.1.1.3 Bacterial strain. The test strain, *Salmonella typhimurium* strain was purchased from Difco Laboratories (Detroit, MI, USA). *Salmonella typhimurium* stock cultures were stored at –80 ^{o}C and fresh sub-cultures from frozen stock were prepared on a regular basis. Active cultures were transferred to fresh media every week.

3.1.1.4. Inoculations of the bacteria on the nutrient agar plates. An overnight culture (12hrs) was used for serial dilution. After measuring the optical density (O.D) at 600nm the culture was serially diluted with trypticase soy broth to final concentration of

10^4 cells/mL. The diluted culture was used for inoculation of tryptic agar plates. From the selected tube 50μL of the culture was spread using glass beads.

3.1.1.5. Exposure to neat compounds. After inoculation plates were placed open in the glass desiccator with the lid closed. The specified amount of the neat compounds was injected through the outlet in the lid of the desiccator. Studies were done with inoculated plates exposed to 5, 10, 15, 30, 45, 75, 90, 120 minutes. After exposure plates were incubated for 24hrs at 37 ^oC on a bench top incubator.

3.2 DISINFECTANT ACTIVITY OF OIL VAPORS ON BIOFILMS

A bench scale set-up for vaporization of mineral oil and biogenic oil esters was fabricated from components available in the center for Environmental Science and Technology (CEST). The system consisted of a concentric stainless steel reactor tube. The stainless steel reactor had an outer diameter of 1.25cm and length of 40cm. The tube was placed in an electrically heated tubular furnace. The temperature of the furnace was controlled with a feed back type temperature control unit; temperature of the reactor was monitored and controlled with a "J" type thermocouple. Experimental studies were performed at constant temperature of 650 ^oC ($\pm$ 10 ^oC). The air flow was set at 10 L min^{-1} with a mass flow controller and monitored with a rotameter. A reciprocating pump delivered the oil at a constant flow rate of 0.5ml min^{-1} into the reactor. Oil and air were introduced into the reactor tube through a concentric tube arrangement. A schematic of the set-up is shown in figure 3.1.

The oil was subjected to vaporization in the reactor tube and expelled from the tube with air at high temperature. The mixture under goes rapid cooling to the ambient temperature leading to super saturation, resulting in an air stream laden with oil aerosols and volatile organic vapors formed as a result of thermal-oxidation of oil. The oil particle size of the emerging aerosols lies in between 0.1 - 2 µm and gives air-aerosol stream the appearance of a white smoke.

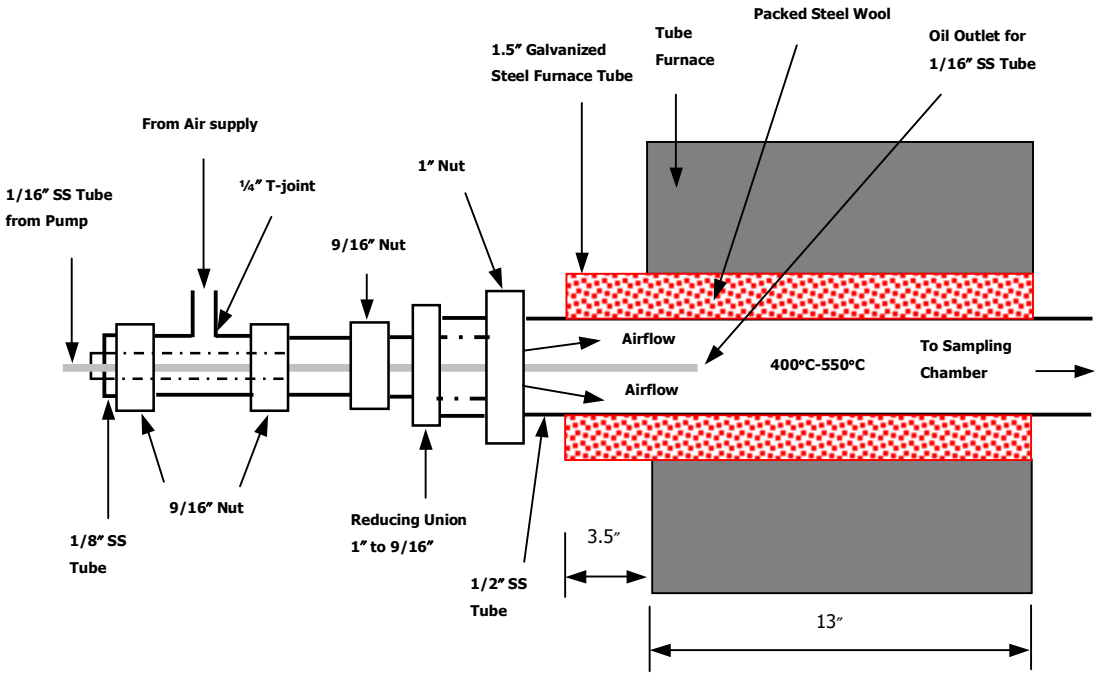


Figure 3.1. A schematic of oil vaporizer set-up. *Adapted from" Evaluations of antimicrobial activities of volatiles produced during rapid volatilization of oils"*

To remove the aerosols from the air stream, a condensation unit and a filtration unit were installed in series with the vaporizer. The condensation unit consisted of a 500 mL conical flask; while the filtration unit consisted of a borosilicate glass tube (30 cm x 5 cm

i.d.) uniformly packed with 60 g of a ceramic wool blanket. The air stream with volatile organics emerging from the filtration unit was split with a "T" connector. The major portion of the aerosol free air stream was passed on to a stainless steel glass exposure chamber (50 cm x 40 cm x 50 cm) and a small portion was sent to a gas tight 350 ml glass sampling bulb. A schematic of the vaporizer with the condensation, the filtration unit, exposure chamber and the gas sampling bulb is shown in Figure 3.2.

The split stream set-up of the generation system allowed simultaneous execution of microbial toxicity assay and chemical characterization of volatile organics. A photograph of the oil vaporization apparatus is shown in Figure 3.3.

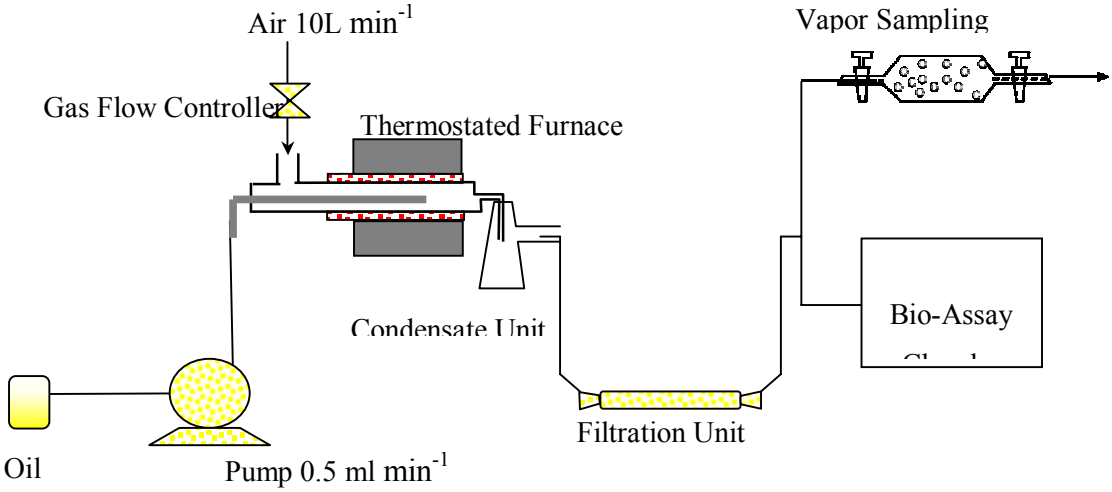


Figure 3.2. A schematic of the bench-scale vapor generation, exposure and gas sampling set-up. *Adapted from" Evaluations of antimicrobial activities of volatiles produced during rapid volatilization of oils"*

Figure 3.3. A photograph of the oil volatilization and condensation set-up. *Adapted from" Evaluations of antimicrobial activities of volatiles produced during rapid volatilization of oils"*

Earlier studies had shown that the volatilization temperature is an important parameter in the generation of volatile disinfectants (51). Optimization of the aerosol generation temperature was done prior to the chemical characterization studies.

3.2.1 Materials. The following materials were used for direct exposure experiments.

3.2.1.1 Oils. Methyl soyate was purchased from AG Environmental Products L.L.C (Lenexa, Kansas).

3.2.1.2 Bacterial cultures of *Klebsiella pneumoniae, Staphylococcus epidermidis* were obtained from Difco laboratories (Detroit, MI) and American Type Culture Collection .Stock cultures were stored at – 80 ^{o}C and fresh sub-cultures from frozen stock

were prepared on a regular basis. Active cultures were transferred to fresh media every week.

3.2.1.3 Bacterial culture media. The following culture media were used for basic bacterial culturing and exposure experiments.

Tryptic soy agar is a nutrient medium suitable for sub-culturing, serial dilutions, and exposure experiments. It was prepared with 1.5% agar and 3.5% tryptic soy broth. 1/10 strength Tryptic soy broth was used for all Biofilm growth.

3.3 GENERATION OF BIOFILMS

Wide-mouth 125ml flasks containing two microscope slides slanted against each other were autoclaved and were filled with 100ml of sterile 1/10 strength tryptic soy broth. *K.pneumoniae* or *S.epidermidiae* were grown to an optical density of 0.5 (2-4 hrs). This density approximately contains 1 x 10^8 cells/mL. 10 µL of the culture was added to the biofilm flask which gave approximately 1 x 10^6 cells/mL in the flask. Inoculated flasks were incubated in a shaker at room temperature for 24, 48 and 72 hrs at 37 ^oC and 100rpm.

3.4 EXPOSURE TO METHYL SOYATE VAPORS

After incubation microscope slides were removed from the flasks with sterile forceps. Carefully one of the slides was removed from the flask and placed it in a sterile petridish. Plates were then placed in the chamber with methyl soyate vapor generated at 650 ^oC. Microscope slides were exposed to smoke for 15, 30 and 45 minutes respectively. After exposure, the biofilm was transferred to sterile broth by scraping with a sterile rubber policeman. The broth containing the biofilm bacteria was spotted on a trypticase

soy agar plate and incubated at 37 ^{o}C for 24hrs. All the experiments were performed in duplicate.

3.5 OPERATION OF SMOKE GENERATOR

The tubular furnace was equilibrated to a preselected temperature of 650 ^{o}C as the vapor generated at this temperature was determined in pervious studies to be most effective in inhibiting bacterial growth. The air was introduced into the stainless vaporization tube. The oil was introduced at set flow rate of 0.5 mL/min with a reciprocating piston pump.Temperature inside the stainless tube was maintained within ± 5 °C of the set point. Efficient aerosolization of oil was indicated by the appearance of a dense white cloud at the exit of the vaporization tube which was filtered using glass wool to separate vapor from the smoke. The slides were placed in the exposure chamber which is a gas-tight stainless steel – borosilicate glass chamber that was placed inside a fume hood. The plates with slides were placed inside and retrieved from the chamber through a hinged door.

3.6 DISINFECTANT VAPOR GENERATOR CLEAN-UP

Components of the disinfectant vapor generation system can act as sources of contamination during analysis. Therefore the oil container, the stainless steel reactor and fittings associated with it were cleaned thoroughly. Components that required cleaning between applications include, oil condensate flask, tygon tubing associated with various components of the generator, ceramic wool blanket, borosilicate glass tube and container for ceramic wool blanket. A thorough cleaning of these components was carried out after each experiment to eliminate cross contamination.

All electrical appliances like heater, oil pump, etc. was switched off. The internal temperature thermocouple attached to the reactor was removed. A rifle barrel cleaning kit was used to clean the reactor to remove any residual condensate matter or carbon deposits from the inner walls. Later, the reactor was rinsed with water, acetone and hexane respectively. The temperature probe was wiped with cleaning wipes without any solvent. The condensate collection flask was thoroughly cleaned with soap water followed by acetone and hexane. All tygon tubing associated with the system was replaced after every use. The used ceramic wool blanket was discarded and replaced with a new one after the container tube had been washed with soap water and rinsed with acetone and hexane.

The tubing associated with the oil pump was rinsed with acetone and subsequently rinsed with oil to be used. This procedure eliminated cross-contamination due rancidity of old oils. The reactor was reinstalled in the heating furnace. Proper functioning of the temperature probes, controllers, and oil pump was checked. Oil and air supply lines were connected. The temperature controller is set to desired setting.

3.7 DETERMINATION OF HEAD SPACE EQUILBRIUM USING GC-FID

Gas chromatography was performed with equipped Model Finnigan Focus GC, (Thermo Electron Corporation instrument, Walnut Creek, CA) equipped with a Flame ionization detector (GC-FID). The apparatus was set-up to be used for quantitative analysis of the head space concentration of the aldehydes tested in the earlier studies.

3.7.1 Chemicals. High purity standards (95-98%) of the major components detected in the aerosol of Methyl soyate were purchased from Sigma Aldrich were selected for analysis. Identification of the constituents was based on retention time matching with their standards.

3.7.2 Standards preparation. A 22.5 mL vial was purged with nitrogen and sealed air tight with cap. Before introducing the neat compound, the vial was weighed. Followed by injection of 5µL of neat compounds and was again weighed to find the weight of the 5µL neat compound.

3.7.3 Vapor generation and Sampling Procedures. The following protocol was used for vapor generation and transfer of sample aliquot vapor into GC. An aluminium desiccator with volume of 6.3 liters was used as the exposure chamber. Two outlet bulk head openings with septum were installed through which neat compound were injected into the desiccator and to retrieve vapor from the desiccator. One septum was installed on the lid of the desiccator and other on the wall of the desiccator. The desiccator flanges were sealed with a gasket and clamps to prevent any loss of vapor. The desiccator was kept in the incubator at 37 ^{o}C while experiments when performed in order to maintain ambient temperature. An aliquot of the neat compound was injected in the desiccator and aliquots of vapor were withdrawn at regular intervals with a gas tight syringe and injected in the GC-FID for analysis.

3.7.4 Instrument parameters. The GC parameters used during this analysis are listed in Table 3.2.

Table 3.2 Gas – Chromatography Parameters for GC-FID system.

Column	Fused silica capillary DB-Wax 30m long x 0.25mm inner diameter x 0.25µm film thickness
Injection	Split less
Injector	230°C
Detector	230°C
Oven temperature	65°C initial; 1minute 10°C/minute to 150°C; 10minutes final hold
Carrier Gas	Helium Flow rate: 1.2 ml/min
FID flame	Hydrogen flow: 30ml/min Air flow: 300 ml/min

Figure 3.4 A photograph of aluminum desiccator with two bulkheads with air tight septum.

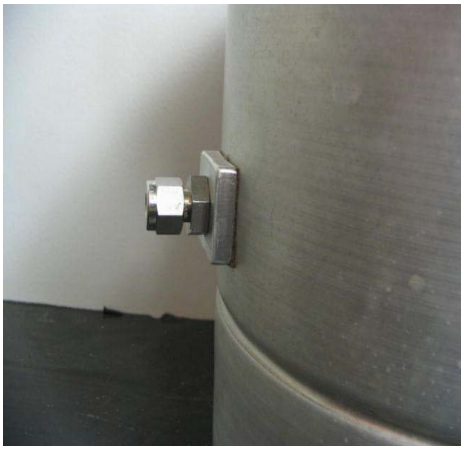


Figure 3.5 A photograph of bulkhead septum valves. On the left is the top inlet valve and right is the bottom outlet valve.

4. RESULTS AND DISCUSSION

4.1 EXPOSURE OF *SALMONELLA TYPHIMURIUM* TO DIFFERENT CONCENTRATIONS OF NEAT COMPOUNDS

4.1.1. Effect of varying concentrations of Hexanal on the bacterial survival.

Trypticase Soy agar plates pre-inoculated with *Salmonella typhimurium* were placed inside the desiccator. Pure hexanal in two different volumes was taken up in a gas chromatography syringe and sprayed into the desiccator through the vacuum inlet. The samples were exposed for different time intervals of 1, 2, 5, and 10minutes and later incubated for 24hrs at 37 ^{o}C. The numbers of colonies were counted after the incubating period. The results are provided in Table 4.1.

Table 4.1. Colony count after exposure to 1µL and 10µL Hexanal at different time intervals.

Concentration	1 Min	2 minutes	5 minutes	10 minutes
Control	112	113	117	112
1 µl	105	105	95	112
10 µl	101	107	107	118

No antimicrobial activity was detected at either time intervals or concentration. Therefore, the same sampling procedure was followed using 50µL hexanal, and the exposure time interval was increased in order to detect the antimicrobial activity of hexanal. The results are provided in the Table 4.2, Figure 4.1 and Figure 4.2.

Table 4.2. Colony counts after exposure to 50µL Hexanal for different time intervals.

Exposure time in minutes	Concentration in µL	Number of Colonies after exposure	Control
30	50	TMTC	TMTC
45	50	300	TMTC
60	50	200	TMTC
75	50	45	TMTC
90	50	No growth	TMTC
120	50	No growth	TMTC

- TMTC= too many to count

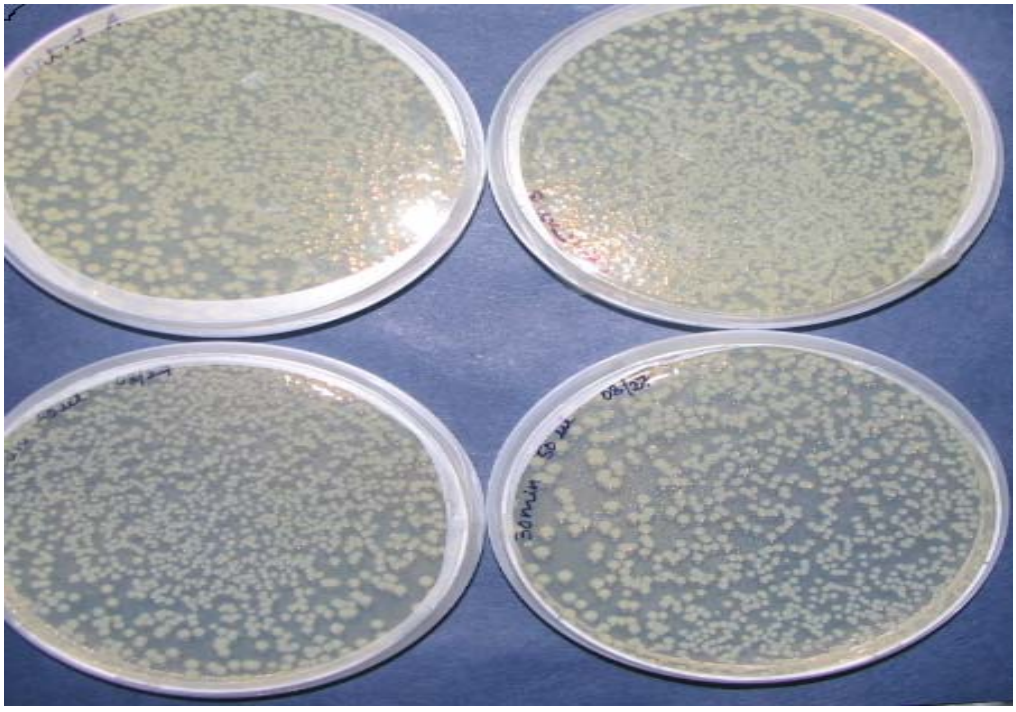


Figure 4.1. A photograph of incubated trypticase soy agar plates after 24hr incubation after exposure to 50µL hexanal. The top row was the no vapor control and in the bottom row 30 minutes exposure plates.

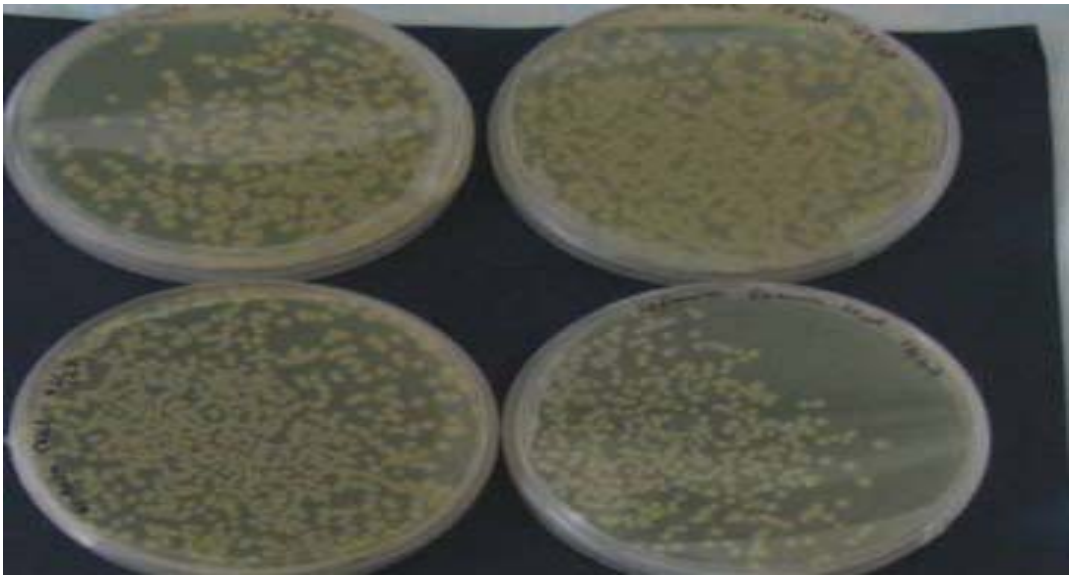


Figure 4.2. A photograph of incubated trypticase soy agar plates after exposure to 50µL hexanal. The top row was 45 minutes exposure and bottom row was after 75 minutes exposure time.

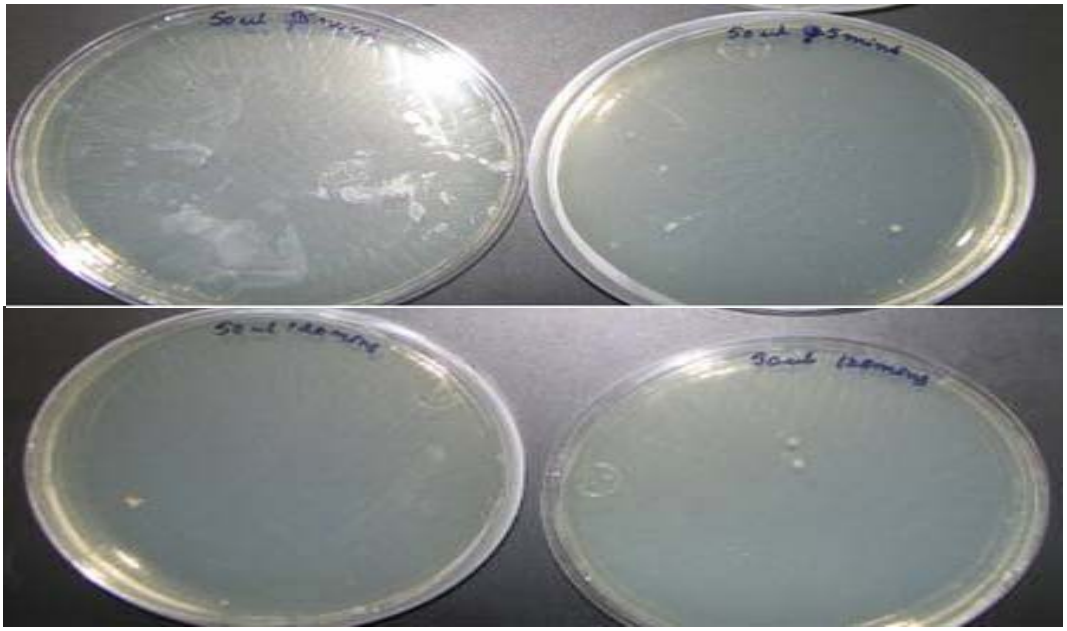


Figure 4.3 A photograph of incubated trypticase soy agar plates agar plates after exposed to 50µL hexanal. The top row was after 90minutes exposure and bottom row was after 120minutes exposure time.

4.1.2 Effect of varying concentrations of Heptanal on the bacterial strains. As with hexanal, 10µL pure heptanal was taken in a GC syringe and sprayed into the desiccator through the vacuum inlet to expose trypticase soy agar plates pre-inoculated with *Salmonella typhimurium*. In addition to varying exposure time, with different cell concentration were also employed. The samples were exposed to different time intervals of 1, 2, and 5 minutes followed by incubation for 24hrs at 37^{o}C. The numbers of colonies were counted after the incubating period. The results are provided in Table 4.3, Figure 4.4 and Figure 4.5 (result provided were for 50µL of 10^{4}cells/mL cell concentration)

Table 4.3 Colony count present after exposed to 10µL Heptanal at different time intervals.

Culture	1 minute	2minute	5 minute
10^{5}cells/ml	TMTC	TMTC	TMTC
Control	TMTC	TMTC	TMTC
10^{4}cells/ml	400	300	200
Control	400	400	400

- TMTC= too many to count

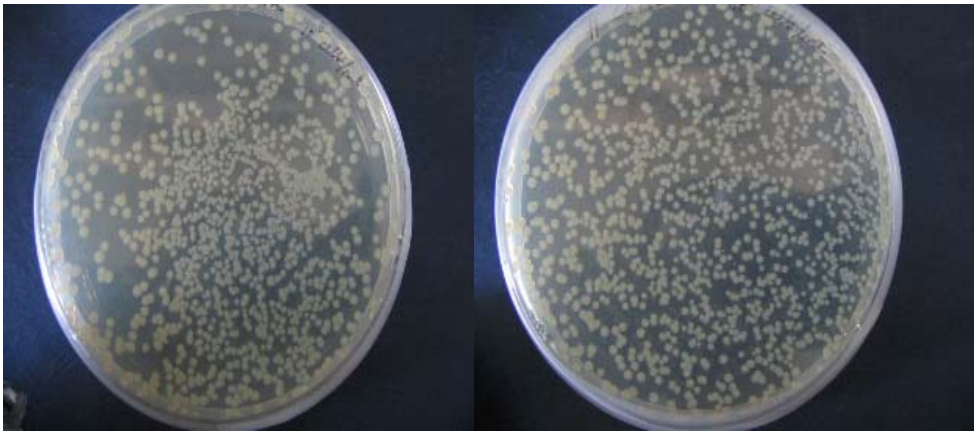


Figure 4.4. A photograph of trypticase soy agar plates incubated after exposed to 10 µL of Heptanal. The left was the no vapor control and on the right was the 1minute exposure time.

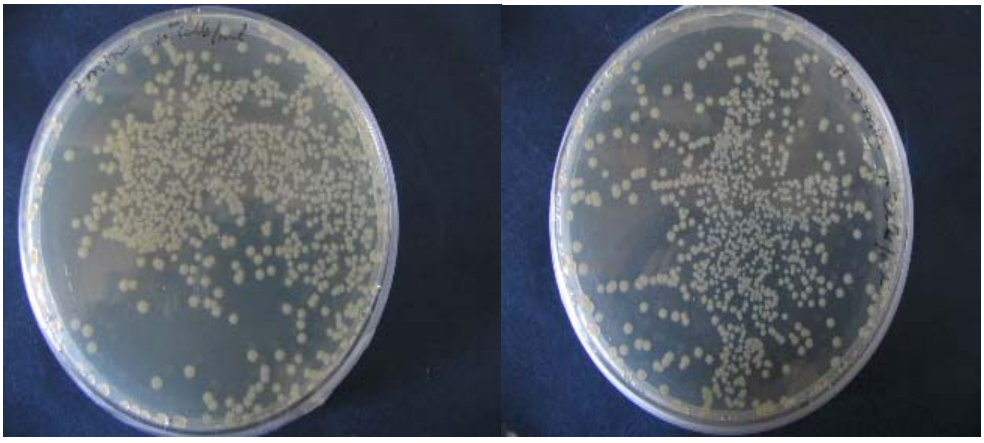


Figure 4.5. A photograph of *Salmonella typhimurium* inoculated trypticase soy agar plate incubated after exposure to 10µL heptanal. On the left was 2 minutes exposure time and on the right is 5minutes exposure time.

Different set of experiments were performed by increasing heptanal concentration and also exposure time. The results are presented below in Table 4.4, Figure 4.6 and 4.7 for

these experiments, 50 µL heptanal were used the trypticase soy agar plates were exposed for 75, 90 and 120minutes.

Table 4.4 Colony count of bacterial strains after exposed to 50µL of heptanal

Concentration	75 minutes	95minutes	120 minutes
50 µL	No growth	No growth	No Growth
Control	TMTC	TMTC	TMTC

- TMTC= too many to count.

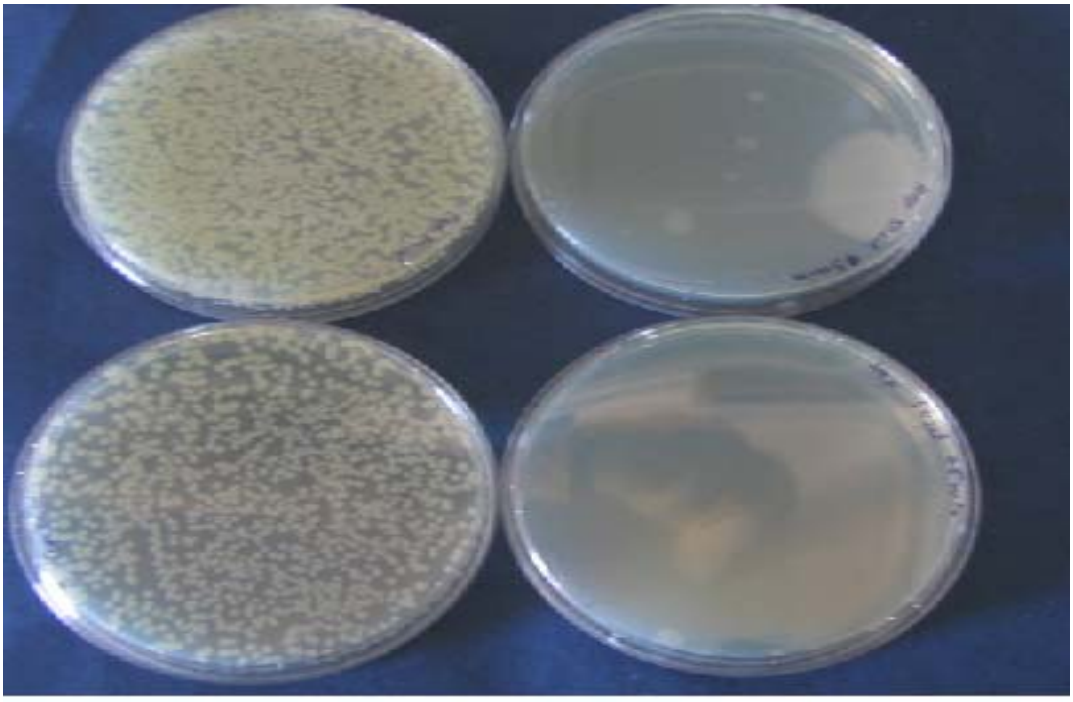


Figure 4.6 A photograph of trypticase soy agar plates incubated after exposure to 50µL heptanal. On the left column was the unexposed and on the right column was 75 minutes exposure time.

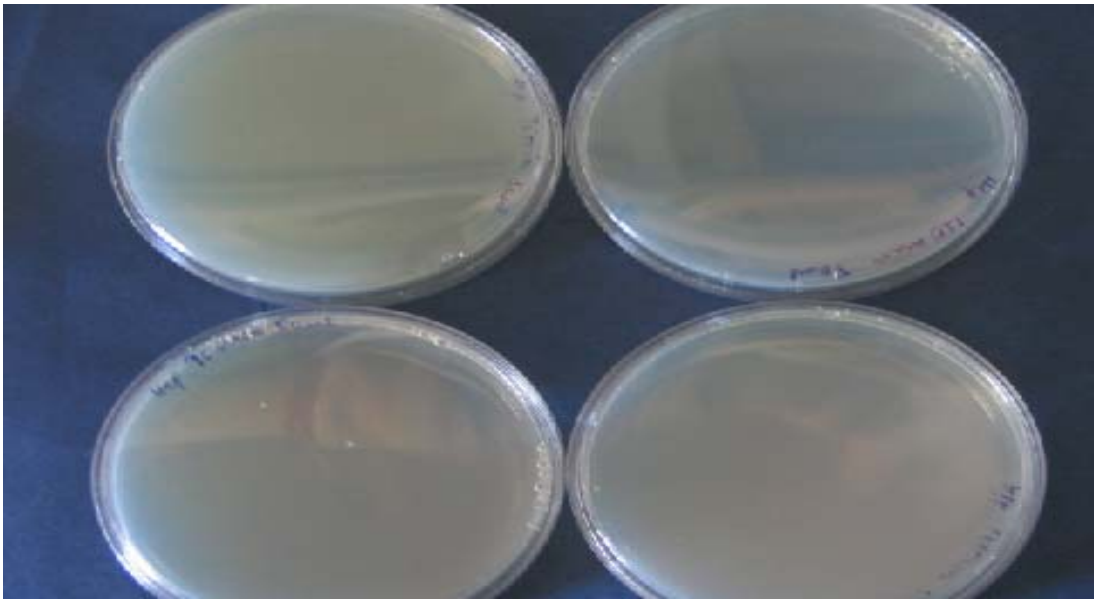


Figure 4.7 A photograph of trypticase soy agar plates after exposed to 50µL heptanal. The left column shows the 90minutes exposure plates and right column 120minutes exposed plates.

4.1.3 Effect of varying concentrations of Pentanal on the bacterial strains. 50µL pure pentanal was sprayed into the desiccator through the vacuum inlet to expose trypticase soy agar plates pre-inoculated with S*almonella typhimurium*. The samples were exposed to time intervals of 75, 90 and120 minutes followed by incubation for 24hrs at 37^{o}C. The numbers of colonies were counted after the incubation period. The results are provided in the Table 4.5 and in Figures 4.8 and 4.9.

Table 4.5 Colony count of bacterial strains present after exposure to 50µL pentanal

Concentration	75 minutes	95minutes	120 minutes
50µL pentanal	175	210	255
Control	TMTC	TMTC	TMTC

- TMTC= too many to count

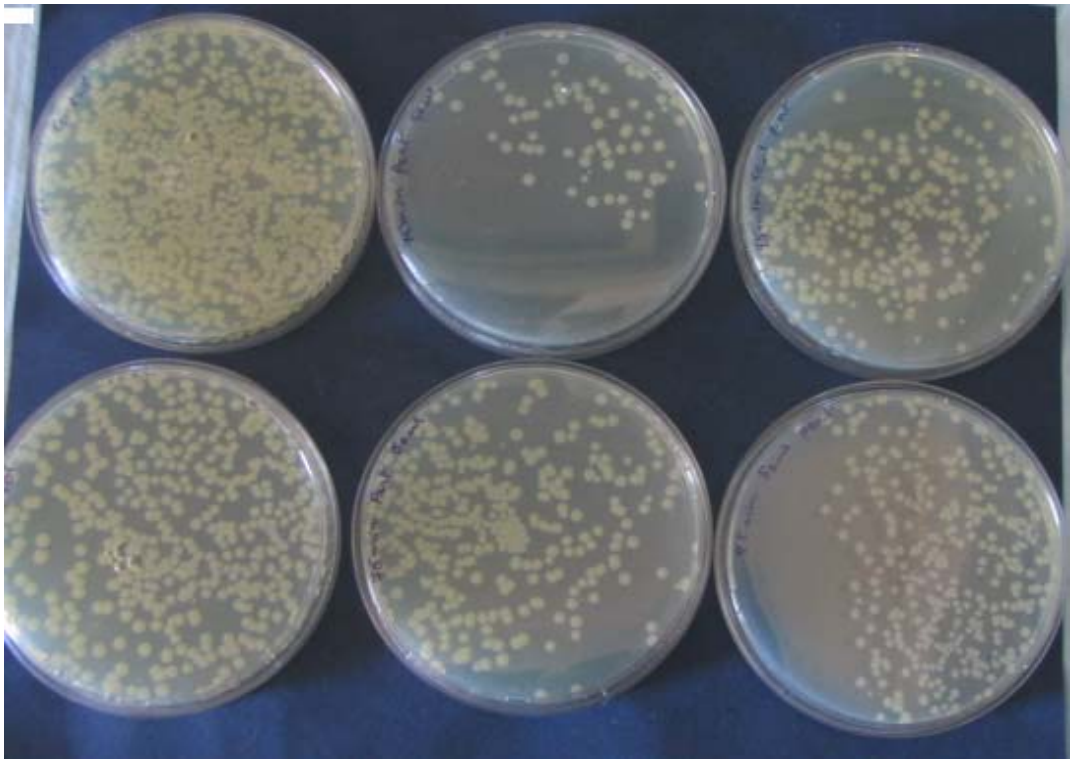


Figure 4.8 A photograph of incubated trypticase soy agar plates after exposure to 50µL of pentanal. Left column was the control plates, in the middle was 75minutes exposure plates and right column was 90minutes exposure plates.

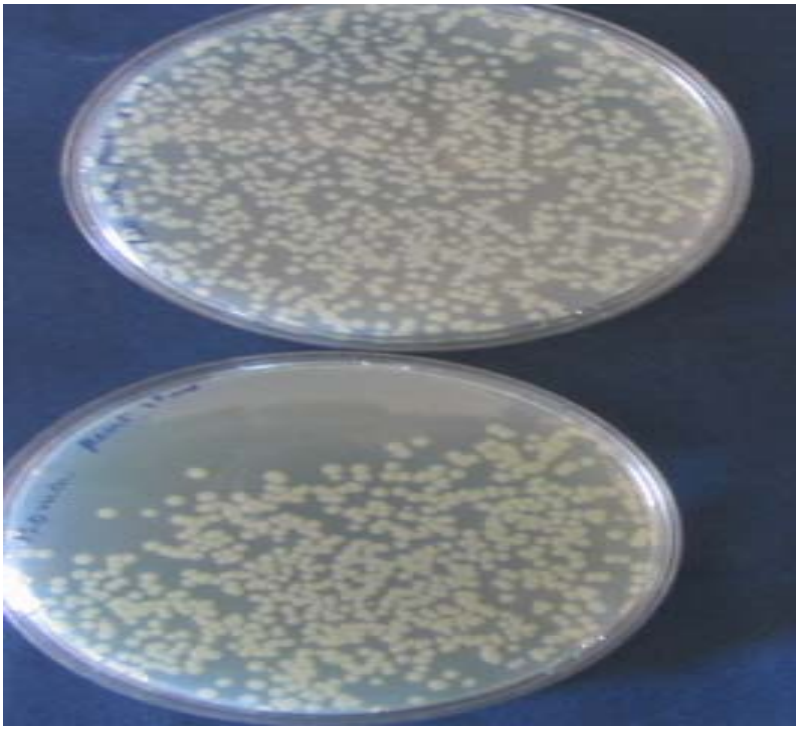


Figure 4.9 A photograph of incubated trypticase soy agar plates after exposure to 50µL of pentanal for 120minutes

4.1.4 Effect of varying concentrations of Propanal on the bacterial strains. Trypticase Soy agar plates pre-inoculated with *Salmonella typhimurium* were placed inside the desiccator. 50µL of pure propanal was taken up in a gas chromatography syringe and sprayed into the desiccator through the vacuum inlet. The samples were exposed to time intervals of 75, 90 and120 minutes and later incubated for 24hrs at 37^{o}C. The numbers of colonies were counted after the incubation period. The results are provided in the table 4.6 and in Figure 4.10 and 4.11.

Table 4.6 Colony count of bacterial strains after exposed to 50µL Propanal.

Concentration	75 minutes	95minutes	120 minutes
50µL Propanal	400	350	300
Control	TMTC	TMTC	TMTC

- TMTC= too many to count

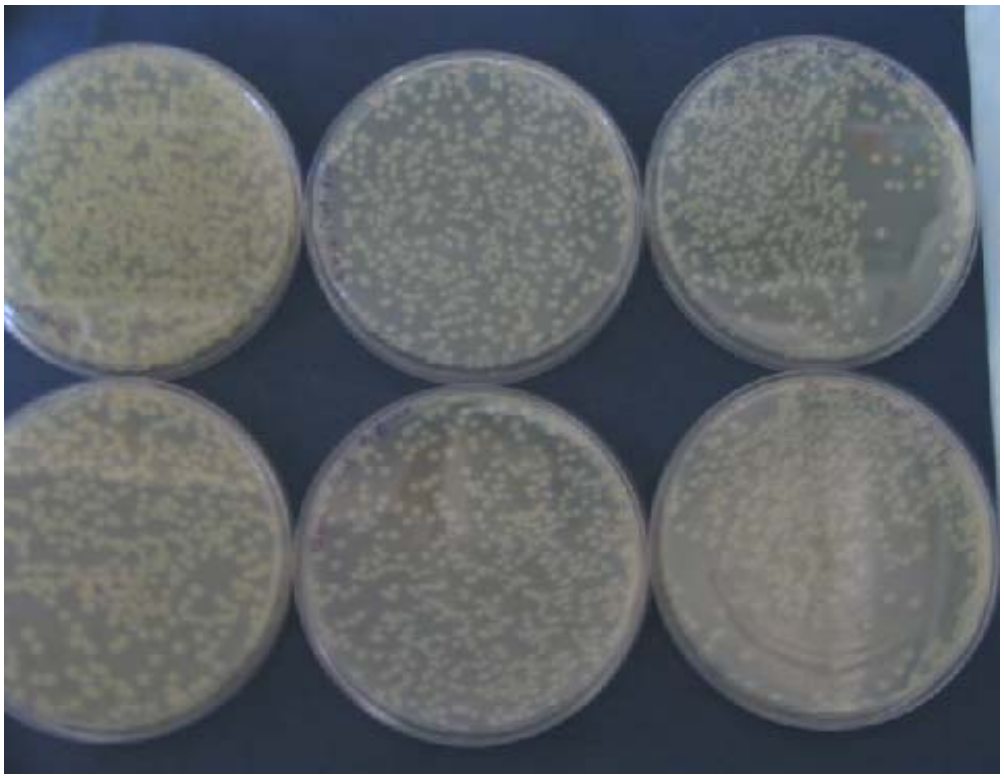


Figure 4.10 A Photograph of incubated trypticase soy agar plates after exposure to 50µL of Propanal. Left column control plates, in the middle 75minutes exposed plates and right column 90 minutes exposed plates.

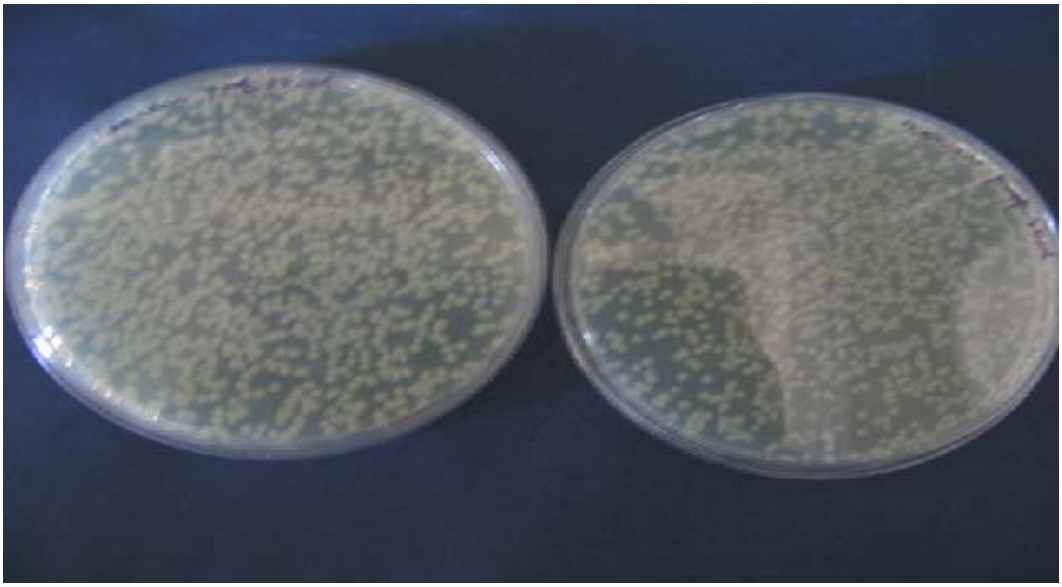


Figure 4.11 A Photograph of incubated trypticase soy agar plates after exposure to 50µL of propanal for 120minutes.

The results of the tests presented above clearly illustrate that hexanal, heptanal, pentanal and propanal (aldehydes which are the major components found in aerosol released during rapid volatilization of methyl soyate) kill S*almonella typhimirium*. However, the concentrations at which the pure samples of the aldehydes possess antimicrobial activity were very high relative to the concentrations detected in analysis of methyl soyate vapors. A summary of the analytical report of methyl soyate aerosol chemical composition and concentration used in our analysis is listed in Table 4.7

Table 4.7 List of compounds worked with and their concentration in comparison with analytical report.

Compounds	Concentration(µg/L) found in analytical report	Concentration (µg/L) of 50µL aldehydes studied for antimicrobial activity
Propanal	108.40µg/L	9088.8µg/L
Pentanal	65.21µg/L	9044.4µg/L
Hexanal	160.80µg/L	8866.6µg/L
Heptanal	79.42µg/L	9000µg/L

Antimicrobial activities of the aldehydes presented in the tests are also time dependant. In Table 4.2 it is shown that increased exposure time from 30 to 120minutes led to increase antimicrobial activity. A comparative summary of the data was provided in Tables 4.8 and 4.9.

Table 4.8. Individual Aldehydes (50µL each) activity at different interval

Compounds	75minutes	90minutes	120minutes
Hexanal	—	—	—
Heptanal	—	—	—
Pentanal	+	+	+
Propanal	+	+	+
Formaldehyde	—	—	—
Control	+	+	+

Table 4.9 Hexanal and Heptanal with two different concentrations individually.

Compounds	75minutes	90minutes	120minutes
Hexanal 20ul	+	+	+
Hexanal 35ul	+	+	_
Heptanal 20ul	+	+	+
Heptanal 35ul	+	+	PG
Control	+	+	+

- PG = Partial growth

4.1.5 Disinfectant activity of mixed Aldehydes. Because the neat compounds did not appear to have the expected antimicrobial activity, it is possible that the strong antimicrobial activity of the oil vapors is due tot he synergistc effects of the compounds. Therefore, some of the compounds were tested in the combination to look for the synergistic effects. Pure hexanal and heptanal same amount were taken in equal proportion with two different syringes and injected into the desiccator through the vacuum inlet with trypticase soy agar plates pre-inoculated with *Salmonella typhimurium* placed inside the desiccator. The samples were exposed for different time intervals of 75, 90 and 120 minutes and later incubated for 24hrs at 37 ^{o}C . The numbers of colonies were counted after the incubation period. The results are provided in the Table 4.10 below.

Table 4.10 Hexanal and Heptanal mixed in equal proportion.

Compounds	75minutes	95minutes	120minutes
Hexanal (25µL) Heptanal (25µL)	+	—	—
Hexanal (15µL) Heptanal (15µL)	+	+	+
Hexanal (12.5µL) Heptanal (12.5µL)	+	+	+
Control	+	+	+

4.1.6 Disinfectant activity of Aldehydes and ketones mixture. Pure aldehydes and ketones (listed earlier in Table 3.4) each at 10 times the concentration found in Methyl soyate vapor were mixed to detect the synergistic effects. Antimicrobial activity of the total mixture i.e., 6.4 µL and 13 µL were tested. The calculated amount of the mixture was taken up in a syringe and sprayed into the desiccator through the vacuum inlet of the desiccator where trypticase soy agar plates pre-inoculated with *Salmonella typhimurium* were placed inside the desiccator. The samples were exposed to different time intervals of 75, 90 and 120 minutes and later incubated for 24hrs at 37 ^{o}C. Calculations are provided in the Table 4.11 The numbers of colonies were counted after the incubation period. The results are presented in the Table 4.12.

Table 4.11 Calculations for converting µg/L to µl/L of the analytes used for the mixed aldehydes and ketones study.

Compounds	Concentration in µg/L	X = 10x µg/L	Density	In µl/L
Acetaldehyde	25.71	257.1	0.785	0.327
Propanal	108.40	1084.0	0.798	1.358
Butanal	60.29	602.9	0.8	0.7512
Petanal	65.21	652.1	0.81	0.805
Hexanal	160.80	1608.0	0.814	1.975
Heptanal	79.42	794.2	0.818	0.971
2-Hexanone	12.63	126.3	0.812	0.155
2-Heptanone	12.14	121.4	0.82	0.149
Total		5245µg/L		6.4912 µL/L

Table 4.12 Colony count of bacterial strains after exposure to aldehyde and ketones mixtures at different concentrations

Concentration	75mins	90mins	120mins
13 µL	TMTC	TMTC	PG
6.4 µL	TMTC	TMTC	TMTC

- TMTC = too many to count.
- PG = partial growth

The results presented indicate that concentration of the aldehydes used the study were higher when compared to the concentration found in analytical data of vapor. At this point were not sure how much compound taken in liquid form actually vaporized to reach a saturation point at which compound would come in contact with bacterial colonies to show antimicrobial activity. And also studies with propanal and pentanal showed no antimicrobial effect at higher concentration, from this it was concluded that the antimicrobial activity of the methyl soyate vapor might due to the synergistic effect of all the chemical components in the vapor. After testing with aldehydes and ketones mixture each at 10 folds the concentration found in methyl soyate vapor no antimicrobial activity was observed indicating that it is necessary to know the headspace concentration of the individual aldehydes which would explain the amount of liquid compound actually in vapor phase at 120 minutes time interval.

4.2 DETERMINATION OF HEADSPACE EQUILIBRIUM

In a pre-cleaned desiccator aliquots of hexanal and heptanal were added and immediately 50µL of the vapor was withdrawn and injected into GC-FID. Vapors were

collected at regular intervals from 0, 15, 30, 45, 60, 75, 90, 105, 120, 135 and 150minutes respectively. Retention time for hexanal and heptanal was determined using standards and sample retention was compared with standards. Retention time chromatographs for standard hexanal and heptanal are provided in Figure 4.12 and Figure 4.13. Graph 4.1 and Graph 4.2 of hexanal and heptanal peak area verses time was plotted.

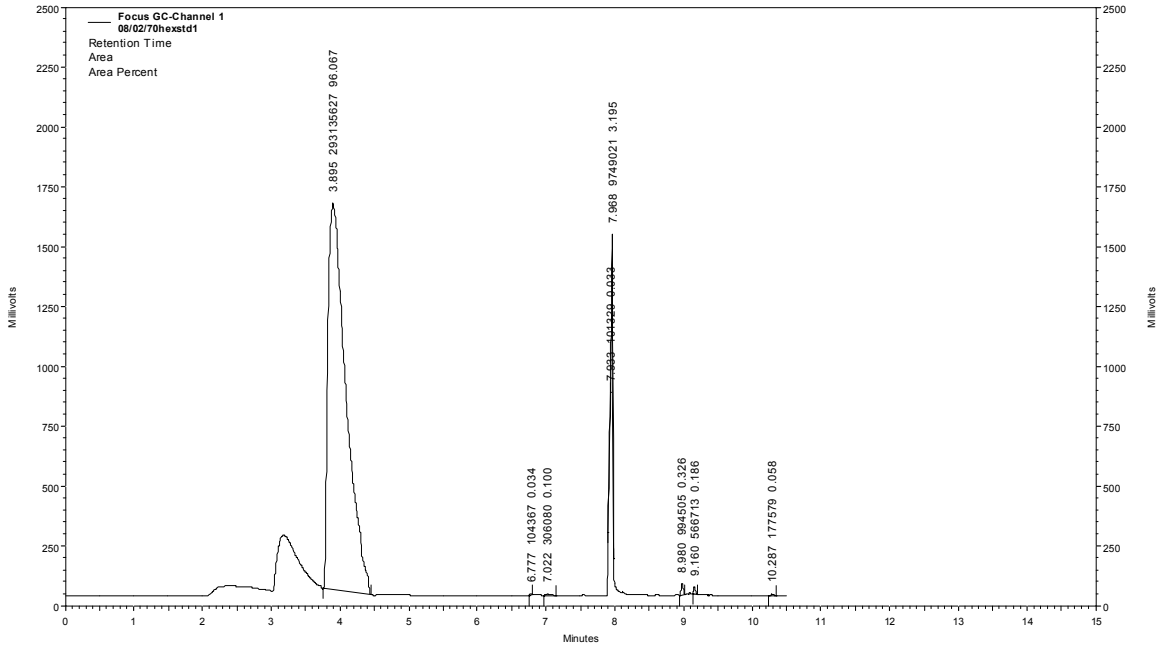


Figure 4.12. A chromatograph of Hexanal standard with retention time at 3.9minutes.

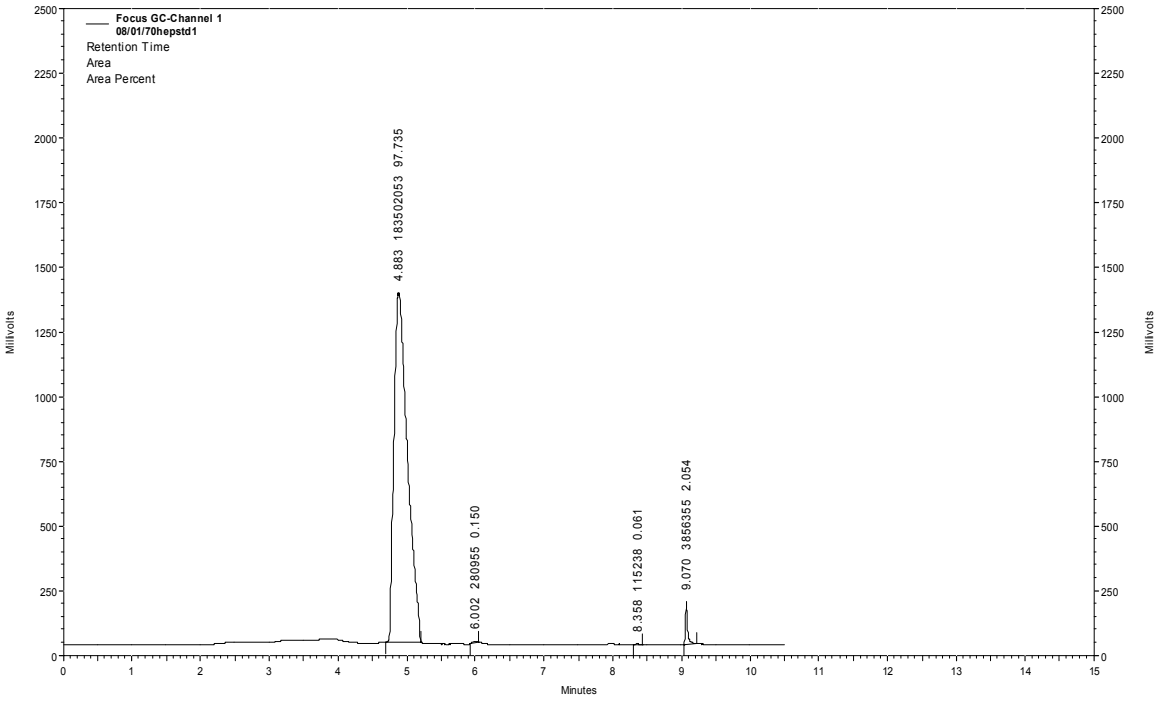
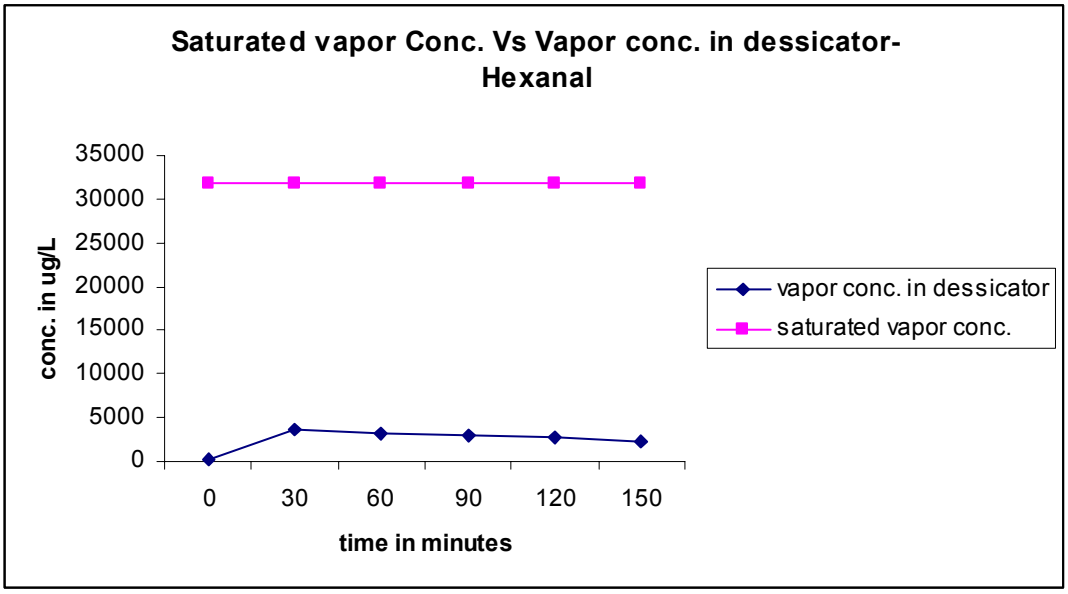
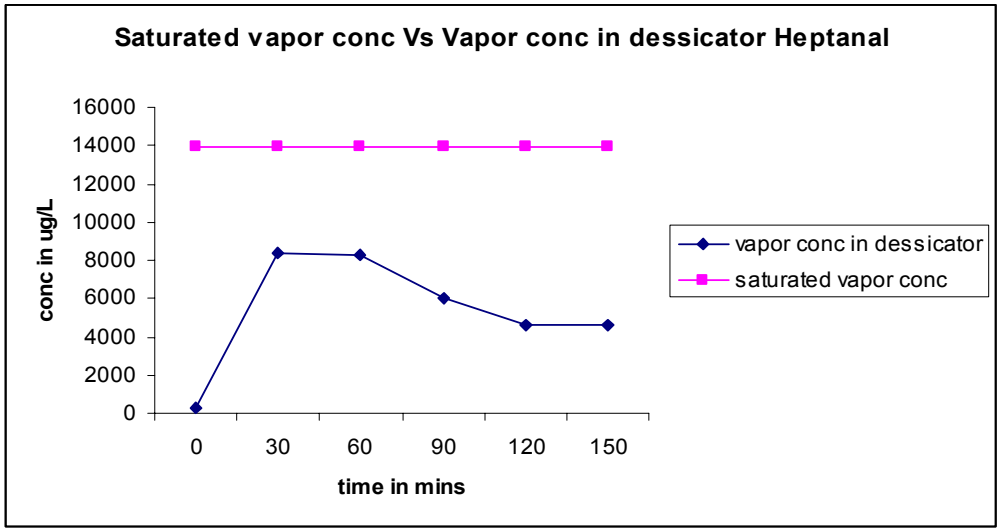


Figure 4.13 A chromatograph of Heptanal standard with retention at 4.9minutes.



Graph 4.1. A graph representing hexanal concentration versus time.



Graph 4.2 A graph representing heptanal concentration versus time.

Amount of hexanal and heptanal used for the study were 50µL (6460.34µg/L for hexanal and 6492.0µg/L for heptanal) and volume of the desiccator in which experiments were conducted was 6.3liters. Several aliquots of 50µL vapor was taken from the desiccator at 15 minutes time intervals, it was observed from the experimental data obtained for both hexanal and heptanal that the concentration of aldehydes per liter in vapor phase increased for the first 45 minutes and gradually reduced. It was observed for both hexanal and heptanal the vapor reached equilibrium at 120 minutes. The amount of hexanal present at saturation point i.e., at 120 minutes was 2676 µg/L (0.13µg in 50µL hexanal vapor) and for heptanal was 4626 µg/L (0.2313µg in 50µL heptanal vapor). The amount of hexanal present at 120minutes was the amount where antimicrobial activity was observed which indicates that only 1/3 of the actual concentration of hexanal (liquid phase) which was 20.71µL (amount in 6.3lts of desiccator) used was effective. For

heptanal the effective concentration where antimicrobial activity was observed is 4626µg/L i.e., 35.6µL which was than the actual amount used. The amount of hexanal and heptanal used for the disinfectant activity study was very high in comparison with the amount found in the methyl soyate smoke analytical data. However the effective concentration of the liquid aldehydes in vapor phase was less, as the initial concentration of the hexanal taken was 50µL and the amount of compound found at 120 minutes time interval was 20.71µL and 35.6µL indicating antimicrobial activity was achieved at lower concentration than the concentration of the liquid aldehydes used.

From the graphs decreased in the vapor concentration as time increase could be due to adherence of the compounds to the walls of the desiccator, leakage from the desiccator and escape of the vapor from syringe while transferring vapor from desiccator to GC-FID.

4.3 DISINFECTANT ACTIVITY OF METHYL SOYATE ON BIOFILMS

Methyl soyate vapors were generated at 650 ^{o}C with the help of smoke generator. Biofilms used in the study were from 24hrs, 48hrs and 72hrs bacterial cultures. The two strains used were *Klebsiella pneumonia* and *Staphylococcus epidermidis.* Biofilms were generated on glass microscope slides, and later exposed to MS vapor for 15, 30 and 45mins. Exposed glass slides were scraped with rubber policeman and spotted on trypticase soy agar plates which were incubated for 24hrs at 37 ^{o}C.

The results for the tests are provided in the Tables 4.13, 4.14 and 4.15.

Table 4.13 Colony count of bacterial colonies from 24hrs biofilms after exposure to MS derived vapor.

Strains	15 mins	30mins	45mins
Staphylococcus epidermidis	—	—	—
Klebsiella pneumonia	—	—	—
Control	+	+	+

Table 4.14 Colony count of bacterial colonies from 48hrs biofilms after exposure to MS derived vapor.

Strains	15 mins	30mins	45mins
Staphylococcus epidermidis	—	—	—
Klebsiella pneumonia	—	—	—
Control	+	+	+

Table 4.15. Colony count of bacterial colonies from 72hrs biofilms after exposure to MS vapor.

Strains	15 mins	30 mins	45 mins
Staphylococcus epidermidis	—	—	—
Klebsiella pneumonia	—	—	—
Control	+	+	+

The above presented results show how effectively Methyl soyate vapors kill biofilms from *Staphylococcus epidermidis* and *Klebsiella pneumonia*. Biofilms major impacts on human health and industries including petroleum, specialty chemicals, health, household products, drinking water, mining, utilities and medical devices they also resist to several potent antibiotics. Earlier work with vapors generated from methyl soyate a biogenic oil ester showed potential disinfectant against wide range of bacteria which are responsible for various contaminations include *Pseudomonas aeruginosa*, *Enterococcus cloacae*, *Escherichia coli, Serratia marcescens, Klebsiella pneumoniae* and *Salmonella typhimurium.* The vapors were lethal to the bacteria infecting the inoculated building materials these experiments were done using *Enterobacteriaceia* HYChecks and complete lethality was achieved when the concentration is 10^4CFU/100mL. Vapors was also proved to be a effective sporicidal on bacterial endospores of *Bacillus sublitis* and *Bacillus stearothermophilus*. Methyl soyate vapors are more efficient, easy post disinfection cleanup, low toxicity to higher organisms, and low corrosiveness are few of its observed advantages compared to presently employ potent disinfection techniques.

CONCLUSION

Earlier studies, conducted at the Center of Environmental Science and Technology- University of Missouri, had shown that methyl soyate when subjected to thermal oxidation at elevated temperature produces volatiles chemicals which are very effective in killing a wide range of Gram-negative and Gram-positive bacteria.

Effectiveness of these volatile chemicals against bio-films was evaluated as part of the studies reported in this thesis. Biofilms were generated over time periods ranging from 24hours to 72 hours and then exposed to the thermal oxidation derived from methyl soyate. In all cases the methyl soyate derived vapors were effective in killing bacteria present in the bio-films. The results prove the anti-bacterial efficiency of the methyl soyate derived chemical.

Others experiments reported in the thesis were directed at evaluations of antimicrobial activities of predominant chemical detected in the thermal oxidation products. Analysis of the methyl soyate leads to volatile oxygenated species including short chain alcohols, aldehydes, ketones and acids. Antibacterial activities of individual chemicals were assessed by exposing *Salmonella* cultures to vapors of selected chemicals. Results of the experiments showed that individual aldehydes such as pentanal, hexanal and heptanal exhibit antimicrobial activity but only at very high concentration relative to those observed in the thermal oxidation products. Even the mixture of aldehydes did not exhibit antibacterial activity at concentration 10times higher than the concentration in the thermal oxidation stream. Results lead to the conclusion that the

antimicrobial activity of the thermal oxidation products stem from the synergistic effects of the compounds and/ or may be related to unidentified chemical/s in the thermal oxidation product stream, A more comprehensive chemical analysis of these products is therefore recommended as part of future studies.

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